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Lipopolysaccharide transport to the cell surface: biosynthesis and extraction from the inner membrane

**Brent W. Simpson¹, Janine M. May², David J. Sherman², Daniel Kahne^{2*},
Natividad Ruiz^{1*}**

¹*Department of Microbiology, The Ohio State University, 484 West 12th Avenue, Columbus, OH 43210,
USA*

²*Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford Street, Cambridge, MA
02138, USA*

³*Department of Biological Chemistry and Molecular Pharmacology, 240 Longwood Avenue, Harvard
Medical School, Boston, MA 02115, USA*

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*Author for correspondence (kahne@chemistry.harvard.edu; ruiz.82@osu.edu).

†Present address: DK: Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford Street, Cambridge, MA 02138, USA; NR: Department of Microbiology, The Ohio State University, 484 West 12th Avenue, Columbus, OH 43210, USA

Summary

The cell surface of most Gram-negative bacteria is covered with lipopolysaccharides (LPS). The network of charges and sugars provided by the dense packing of LPS molecules in the outer leaflet of the outer membrane interferes with the entry of hydrophobic compounds into the cell, including many antibiotics. In addition, LPS can be recognized by the immune system and play a crucial role in many interactions between bacteria and their animal hosts. LPS is synthesized in the inner membrane of Gram-negative bacteria, so it must be transported across their cell envelope to assemble at the cell surface. Over the last two decades, much of the research on LPS biogenesis has focused on the discovery and understanding of Lpt, a multi-protein complex that spans the cell envelope and functions to transport LPS from the inner membrane to the outer membrane. This chapter focuses on the early steps of the transport of LPS by the Lpt machinery: the extraction of LPS from the inner membrane. The following chapter describes the subsequent steps as LPS travels through the periplasm and the outer membrane to its final destination at the cell surface.

Introduction

All cells are defined by a cellular envelope that directly contacts the outside world and whose integrity is required for cell survival. Consequently, the fate of a cell in any given environment is greatly determined by the architecture and composition of its cell envelope. The bacterial cell envelope can contain one or two membranes termed monoderm and diderm respectively. The major type of diderm bacteria are the so-called Gram negatives. The unique structure and composition of the Gram-negative cell envelope allows the survival of these single-cell organisms in many different environments. This mainly depends on the ability of their cell envelope to selectively allow the entry of useful molecules such as nutrients, while preventing the entry of harmful ones including many antibiotics.

The Gram-negative cell envelope consists of two lipid bilayers each with distinct composition and permeability properties. These two membranes are separated by an aqueous compartment, termed the periplasm, and a cell-wall polymer composed of peptidoglycan [1]. The inner membrane (IM), which surrounds the cytoplasm, is a typical phospholipid bilayer, while the outer membrane (OM), which is the outermost cell boundary, is asymmetric with phospholipids in the inner leaflet and lipopolysaccharides (LPS) in the outer leaflet. Most of what is known and what we will discuss about the OM comes from studying *Escherichia coli* and *Salmonella typhimurium*. However, it is unclear how representative this is of most Gram negatives. LPS is an amphipathic molecule composed of lipid A, a core oligosaccharide, and a long polysaccharide commonly called O antigen (Fig. 1). Although the overall structure and properties of LPS are conserved, its chemical composition varies among species [2, 3]. In fact, some bacteria synthesize a shorter version of LPS known as lipooligosaccharide or LOS [2]. In addition to the lipid asymmetry conferred by the presence of LPS (or LOS) in the outer leaflet, the OM is structurally different from the IM in that its integral OM proteins adopt a β -barrel conformation instead of the typical α -helical structure of integral IM proteins. Both LPS and β -barrel proteins contribute to the unique permeability properties of the OM. Molecules of LPS pack tightly in the outer leaflet of the OM and the hydrophilicity of its sugars and charges of its phosphates serve as a barrier to small hydrophobic compounds that normally partition and diffuse through phospholipid bilayers like the IM. In contrast, many small hydrophilic molecules can easily cross the OM by diffusing through the hydrophilic lumen of porins—integral β -barrel proteins that function as nonspecific, ungated pores or sieves [4]. As the IM lacks ungated pores, entry of small hydrophilic molecules through the IM relies on transporters that must recognize them to allow their transport into the cytoplasm. Therefore, many molecules that can diffuse across the IM (small hydrophobic compounds) cannot easily penetrate through the OM because of LPS, and many molecules that can diffuse across the OM (small hydrophilic compounds) cannot easily penetrate through the IM. The fact that these two membranes have opposite permeability properties protects cells from many harmful compounds and is one of the major reasons why it is so difficult to develop antibiotics that are effective against Gram-negative bacteria.

The importance of LPS in providing resistance against hydrophobic antibiotics was revealed from studies of wild-type cells where LPS was removed by chemical treatment or of mutant cells defective in LPS biogenesis [4, 5]. Collectively, these studies demonstrated that decreasing the amount of LPS at the cell surface leads to the appearance or an increase of phospholipids at the cell surface. When phospholipids are present in the outer leaflet, the asymmetry of the OM is broken, effectively creating patches of phospholipid bilayer, which allow the diffusion of small hydrophobic compounds into the cell. Indeed, a decrease in the levels of LPS at the cell surface leads to hypersensitivity to hydrophobic toxic molecules such as bile salts and some antibiotics. Furthermore, these studies also revealed that some bacterial species such as *Escherichia coli*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa* cannot survive without LPS, while others such as *Neisseria meningitidis*, *Acinetobacter baumannii* and *Moraxella catarrhalis* can [6-11]. Therefore, it is crucial that, during growth at each cell cycle, Gram-negative bacteria properly assemble millions of LPS molecules at their cell surface at a speed estimated to be as high as 70,000 molecules/min [3].

For more than 40 years, we have known that although LPS must be present at the cell surface, its synthesis occurs at the IM [12, 13]. In the last two decades, the players involved in the transport of LPS across the cell envelope have been identified. This review and the accompanying piece [14] highlight the research that has led to our current understanding of how, following synthesis at the inner leaflet of the IM, LPS is flipped across the IM, extracted from the IM, transported across the aqueous periplasm, and translocated through the OM so that it can finally assemble at the cell surface (Fig. 1). This chapter focuses on the early steps that take place at the IM in the journey of LPS across the cell envelope. The following chapter will delve into the details of LPS transport across the periplasm and insertion into the OM [14].

Synthesis, transport, and biogenesis of the LPS layer

Electron microscopy studies drove the discovery that the Gram-negative cell envelope is delimited by two membranes [15]. Electron micrographs produced by Kellenberger and Ryter were the first to clearly distinguish that there were three layers in the *E. coli* cell envelope [16]. The chemical composition of these multilayers took some time to describe. Bladen and Mergenhagen found that the outer layer of the Gram-negative *Veillonella* bacterium could be extracted with phenol-water and that the extracted material retained the endotoxic effects associated with LPS [17]. The intermediate layer could be broken down by lysozyme indicating that it was peptidoglycan [17]. Thus it was soon deduced that Gram-negative bacteria have an inner and outer lipid membrane with peptidoglycan in between them. The asymmetric nature of the OM was demonstrated by two seminal studies. Muhlradt and Golecki showed that LPS was on the cell surface by immuno-electron microscopy, while Kamio and Nikaido found that the main OM phospholipid, phosphatidylethanolamine, was not accessible to external modification and thus not on the bacterial surface [13, 18].

Around the same time, studies on the synthesis of LPS led to the ground-breaking realization that LPS synthesis takes place at the IM. The development of fractionation methods by Miura and Mizushima to separate the IM and OM by density allowed Osborn and collaborators to show that although the majority of LPS resides in the OM, it is synthesized at the IM [12, 19, 20]. This study was pivotal, as it provided the first evidence that LPS must be transported across the cell envelope. Muhlradt *et al.* further showed that all synthesis steps occur at the IM before transport [21]. Both of these studies also demonstrated an important point: transport of LPS to the cell surface is irreversible.

Extensive studies on the biosynthesis of LPS, led mainly by Christian Raetz and collaborators, culminated in the elucidation of the synthesis pathway, which revealed that the lipid A-core subunit of LPS is synthesized at the cytoplasmic side of the IM [3, 22]. We now also know that the O antigen is synthesized independently of lipid A-core and that its ligation to the lipid A-core, as well as other LPS modifications, occur at the periplasmic side of the IM [2, 22, 23]. Together, these works broke the ground for studies of the transport of LPS across the cell envelope. They also indicated that LPS localization to the OM requires at least two distinct steps: flipping of lipid A-core across the IM and transport of fully synthesized LPS across the periplasm to the OM. Because most

of the studies on LPS transport across the cell envelope have been conducted in cells that do not synthesize O antigen, we will only describe hereafter the transport of the lipid A-core subunit, which, for simplicity, we will refer to as LPS.

MsbA flips LPS across the IM

Georgopoulos and collaborators discovered the LPS flippase MsbA in a genetic screen as a multicopy suppressor of the temperature-sensitive allele of *htrB* [24]. Discovery that HtrB, renamed LpxL, is a lauroyl acyltransferase involved in the synthesis of LPS led researchers to propose that MsbA was a transporter of lipid A because it belongs to the ATP-binding cassette (ABC) family of proteins [25, 26]. Polissi, together with Georgopoulos, Raetz, and collaborators, quickly followed up by demonstrating that MsbA is involved in transport of newly synthesized LPS to the OM [27, 28]. The function as a flippase was finally verified by Doerrler *et al.* by demonstrating that modification of LPS with aminoarabinose and phosphoethanolamine, which occurs at the outer leaflet of the IM, is dependent on MsbA [29].

The flipping mechanism of MsbA has received ample focus and it has become a model to study structural changes that ABC exporters undergo during transport. As in other ABC transporters, ATP hydrolysis provides the energy for MsbA function [27, 30]. Structural studies have demonstrated that MsbA forms a homodimer with fused nucleotide binding and transmembrane domains (NBD and TMD respectively) [31]. Further, x-ray crystallography has revealed multiple conformations of MsbA including two inward-facing states where the NBDs undergo a large conformational change (Fig. 2A) [31]. A ~50Å movement of the NBDs suggests that these domains readily separate and dimerize possibly to allow substrate to enter the lumen of the transporter [31].

These conformational changes of MsbA have been recently studied in a membrane mimicking environment by single particle electron microscopy that captured MsbA in a continuum of conformations. The separation of the NBDs was consistent with the range reported in crystal structures (40-85Å) and the majority of MsbA molecules without ATP were in a closed to moderately open state (40-60Å) [32]. Furthermore, there is *in vivo* evidence of this conformational change. Using fluorescence resonance energy transfer, Doshi and van Veen have shown that binding of lipid A induces NBDs dimerization and stimulates ATP hydrolysis [33]. Structural studies have also revealed that ATP hydrolysis promotes transition to an outward-facing conformation accompanied by a twisting of the transmembrane domains (Fig. 2B) [31]. This conformational change is thought to insert lipid A into the outer leaflet of the IM. Despite the tour de force of structural work on MsbA, mechanistic details of flipping remain unclear.

Interestingly, MsbA has been implicated as a multidrug transporter as well [34]. Lipid A and amphipathic drugs can bind MsbA at the same time, suggesting that MsbA has separate binding sites for these two types of substrates [35]. These multiple roles in antibiotic resistance and LPS transport make MsbA a great target for future antibiotic design.

Debate over intermembrane structures as a means of transport of OM components from the IM to the OM

Once LPS is flipped by MsbA across the IM, it must be transported across the cell envelope. Transport of lipids and proteins from the IM to the OM could be thought to occur in three biologically relevant ways: vesicular transport, diffusion at sites of fusion between the two membranes, or protein mediated transport. Eukaryotic systems have evidence of all three types of transport mechanisms being involved in lipid transport between organelles [36].

To investigate cell envelope structure and biogenesis in bacteria, early research took advantage of the powerful technique of electron microscopy. No visual evidence of vesicles in the periplasm was ever observed, but some groups reported areas of adhesion between the IM and OM noticeable upon plasmolysis [37]. These were first described by Manfred Bayer for whom they were named *Bayer bridges*. Their description started a long debate about whether or not they were physiologically relevant. Some groups argued that the observed structures are artefacts of certain techniques for fixing cells [38]. Meanwhile, Bayer and colleagues argued that they are sensitive

structures broken in harsher preparations [39]. Some support was given to the transport of LPS through *Bayer bridges* when Muhlradt *et al.* demonstrated that newly synthesized LPS appeared at distinct locations in the OM that often overlapped with *Bayer bridge* structures [21].

We now know that LPS transport occurs through proteinaceous bridges that extend from the cytoplasm to the OM [40-43]. The first evidence of this type of transport was provided by Tommassen and collaborators, who showed that LPS transport can still occur in spheroplasts that maintain IM-OM contacts and that are devoid of periplasmic contents [43]. The proteins involved in LPS transport that form these bridges have been named Lpt and they will be the focus of the remaining discussion of this piece and the accompanying chapter [14]. Whether Lpt bridges correspond to some of the controversial *Bayer bridges* remains unknown.

The Lpt bridge

Lpt proteins (LptA through LptG) transport LPS from the IM to the OM. Although experimentally Lpt has been studied most extensively in *E. coli*, this LPS transporter is thought to be structurally and functionally conserved among LPS producers [44, 45]. The seven Lpt factors form an eight-member multi-protein complex (LptAB₂CDEFG) that spans from the cytoplasm to the OM (Fig. 1). Knowledge about the sub-cellular location of each Lpt protein and the steps required for transport has guided most of the models about how Lpt functions. After LPS is flipped across the IM by MsbA, the cell needs to extract the glycolipid from the outer leaflet of the IM, transport it across the aqueous periplasm and the OM, and assemble it at the outer leaflet of the OM. Consequently, each of these stages in LPS transport have been respectively attributed to those factors localized at the IM, periplasm, and OM.

The IM sub-complex consists of LptB₂CFG and is responsible for the extraction of LPS from the IM. LptB is a cytoplasmic protein belonging to the ABC transporter NBD family that associates as a dimer with IM proteins LptCFG [40, 46, 47]. LptFG are polytopic integral IM proteins that constitute the TMD subunits of the LptB₂FG ABC transporter [48]. LptC has a single transmembrane anchor in the IM and a large periplasmic domain that is structurally homologous to both LptA and the periplasmic domain of LptD [40]. As described below, in our current model, the LptB₂FG ABC transporter harnesses energy from ATP hydrolysis to power the extraction of LPS from the outer leaflet of the IM and load the glycolipid onto LptC.

Transit of LPS through the periplasm is mediated through structurally homologous domains found in LptC, LptA, and LptD. LptA physically connects LptC at the IM and the LptDE sub-complex at the OM [41]. LptA is the only component that completely resides in the periplasm, although LptC can function without its membrane anchor [49, 50]. Kahne and collaborators have proposed that LPS travels as a stream through the Lpt system and that when a new LPS molecule is loaded onto the Lpt bridge by LptB₂FG, it pushes LPS molecules that were already ahead of it, causing forward movement towards the OM [42].

Translocation of LPS across the OM and its assembly at the cell surface are mediated by the LptDE OM sub-complex. LptD has two essential domains: an N-terminal periplasmic domain and C-terminal β -barrel [51]. LptE is a lipoprotein that resides within the lumen of the LptD barrel [51, 52]. As discussed in detail in the accompanying chapter [14], recent studies support a model where LPS arrives at the periplasmic domain of LptD and is guided by LptE through LptD so that it can be delivered at the outer leaflet of the OM [45, 53, 54].

In vivo studies have demonstrated that depletion of any one Lpt protein shuts down transport of LPS in *E. coli*, which leads to the accumulation of large membrane structures presumably protruding from the IM into the periplasm [48, 55]. In addition, the fact that the LPS molecules that accumulate at the IM can be modified with colanic acid by WaaL at the periplasmic leaflet of the IM demonstrates that Lpt functions after MsbA flips LPS across the IM [48, 55]. Together, these data and the biochemical evidence demonstrating that all proteins physically interact to form a complex support the model described in Fig. 1.

Discovery of the Lpt extraction machinery LptB₂FGC

Three decades after realizing that LPS must be transported from the IM to the OM, the players involved began to be identified. The Polissi group identified the first IM components of the Lpt system using a genetic screen designed to find essential genes. Serina *et al.* identified one essential operon that contains *yrbG* (still uncharacterized), *kdsDC* (Kdo biosynthesis genes, at the time named *yrbHI*), and *lptCAB* (at the time named *yrbK* and *yhbNG*, respectively) [56]. These studies showed that at least *lptAB* were essential.

In follow-up studies, Polissi and collaborators established the role of LptAB in LPS transport. Because altered expression of *lptAB* can lead to increased sensitivity to hydrophobic toxic compounds or death, they proposed that these factors were essential proteins involved in OM biogenesis [57]. Just as decades before the use of ultracentrifugation to separate the IM and OM led to the discovery that radiolabelled, newly synthesized LPS must be transported to the OM after its synthesis at the IM, this analytical technique was crucial to demonstrate that depletion of either LptA or LptB leads to accumulation of newly synthesized LPS at the IM [58].

Noting these phenotype were similar to those of depleting LptDE, the Polissi and Silhavy laboratories joined forces to demonstrate that, indeed, depletion of each LptABDE and newly characterized LptC resulted in the same phenotypes[55, 58-60]. This suggested that Lpt factors function together. Having identified an Lpt protein in every compartment of the cell suggested a model where LptB was a cytoplasmic ATPase that provided the energy source for transport, LptA was a chaperone that shuttled LPS across the periplasm, and the OM complex of LptDE functioned as a receptor/insertase for LPS.

Sequence analysis clearly predicted LptB as the NBD of an ABC transporter; however, the identity of its TMD partners was uncertain because the IM protein LptC only has a single transmembrane helix and ABC transporters typically have eight to twelve transmembrane helices [61]. Identification of the missing TMDs was accomplished by Ruiz *et al.* through a reductionist bioinformatics approach that took advantage of the essentiality of LPS transport. The group searched for predicted envelope proteins shared by *E. coli* and an endosymbiotic, LPS producing relative that has undergone massive reduction of its genome size[48]. Among the resulting proteins, they searched for those predicted to be essential and of unknown function in *E. coli*, and they identified YjgPQ [48]. Because each was predicted to have a 6-transmembrane helical structure typical of ABC transporter TMDs, and their depletion resulted in phenotypes that resembled those reported for the depletion of Lpt factors, YjgPQ were renamed LptFG and proposed to be the missing TMD of the LptB₂FG ABC transporter [48].

Function of Lpt at the IM: energetics of transport

Without the Lpt complex, LPS accumulates at the outer leaflet of the IM; therefore, the first step in LPS transport is the extraction of the glycolipid from this leaflet (Fig. 1) [48, 55]. LptFGC are positioned to be the likely extractors since LPS resides in the outer leaflet of the IM; the fact that LptB was predicted to be an ATPase suggested early on that ATP hydrolysis is likely the energy source to power extraction [48, 55]. This model raises the fundamental question of how the energy derived from ATP hydrolysis is transmitted across the IM so that extraction of LPS can occur. Most known ABC transporters translocate substrates across membranes [61]. The fact that this ABC transported extracts a substrate from the membrane suggests a novel mechanism of action.

To begin to answer this question, the organization of the IM complex was first studied by Narita and Tokuda, who overexpressed and copurified the complex using a polyhistidine tag on LptB [46]. Size-exclusion chromatography demonstrated that complexes of LptBFGC have a 2:1:1:1 subunit ratio [46]. This was also in agreement with the mechanism of ABC transporters that usually contain a dimer of NBDs [61]. A further advance in our understanding of the LptB₂FG complex was recently made by the Ruiz and Kahne laboratories. Using a combination of structural, biochemical, and genetic approaches, Sherman *et al.* have shown that LptB variants defective in either ATP hydrolysis or interactions with LptFG cannot support growth, demonstrating that LptB must hydrolyse ATP and interact with LptFG to function in LPS transport [47]. Furthermore, X-ray crystal structures of LptB in ATP- and ADP- bound states have suggested that conformational changes

associated with ATP hydrolysis occur in a groove in the LptB dimer that lines the membrane interface that interacts with LptFG (Fig. 3) [47]. We have proposed that these structural changes reveal the means of coupling ATP hydrolysis by LptB to the extraction of LPS by the IM components [47].

The action of the LptB₂FG ABC transporter is intimately related to the transport of LPS along the periplasmic components of Lpt. So far the energetics of LPS flow has been studied for movement along the Lpt bridge from LptC to LptA. Whitfield and collaborators have co-purified His-tagged LptC with LPS [50]. Addition of LptA to their system resulted in the transfer of LPS from LptC to LptA but not vice versa, suggesting that differences in binding affinity of Lpt factors and LPS could be part of the driving force for LPS transport across the periplasm [50]. More recently, Okuda *et al.* have demonstrated that ATP hydrolysis is required for LPS movement along the bridge [42]. Using site-specific photoactivatable cross-linking in a right-side-out vesicle system, the Kahne laboratory has demonstrated that LPS interacts with specific residues within the hydrophobic grooves of LptC and LptA [42]. Importantly, cross-linking of LPS to LptC and subsequent transfer of LPS from LptC to LptA was found to be dependent on ATP hydrolysis [42]. These results have led to the model presented in Fig. 1, where LPS moves in a step-wise and ATPase-driven fashion from protein to protein across the Lpt bridge.

Mechanism of extraction and unknown roles for LptFG

Currently, the first protein in the Lpt bridge known to interact with the substrate LPS is LptC [42, 50]. Interestingly, the aforementioned study by Okuda *et al.* also demonstrated that LptC cannot extract LPS on its own [42]. Extraction and handing off LPS to the bridge could be thought to occur by one of two mechanisms. In one model, LptFG could interact directly with LPS, performing the extraction function coupled to LptB's ATP hydrolysis. Somehow they would then have to pass LPS to LptC. In this model, both LptF and LptG or just one of them could interact with LPS. The other model proposes that, in an ATP-dependent manner, LptFG somehow stimulate LptC to extract LPS from the IM. At this moment, LptFG are the most poorly understood components of the Lpt system. Additional work is needed to understand their essential function in LPS transport.

Conclusion

A large body of research conducted in the last two decades has finally made great progress in elucidating how the glycolipid LPS is transported from its site of synthesis, the IM, to its final location, the cell surface. This progress was possible by the use of many different *in vivo*, *in vitro*, and *in silico* approaches and the efforts of many laboratories. The following chapter resumes the discussion about the major discoveries that have been made regarding the periplasmic and OM components of the Lpt system. Despite all this progress, more research is still needed to understand the mechanism of transport and assembly of the Lpt bridge, and how the cell coordinates LPS transport with other processes that are required for proper envelope biogenesis [62, 63].

References

- [1] Silhavy, T.J., Kahne, D. & Walker, S. 2010 The bacterial cell envelope. *Cold Spring Harb. Perspect. Biol.* **2**, a000414. (doi:cshperspect.a000414 [pii] 10.1101/cshperspect.a000414).
- [2] Trent, M.S., Stead, C.M., Tran, A.X. & Hankins, J.V. 2006 Diversity of endotoxin and its impact on pathogenesis. *Journal of endotoxin research* **12**, 205-223. (doi:10.1179/096805106X118825).
- [3] Whitfield, C. & Trent, M.S. 2014 Biosynthesis and export of bacterial lipopolysaccharides. *Annu Rev Biochem* **83**, 99-128. (doi:10.1146/annurev-biochem-060713-035600).
- [4] Nikaido, H. 2003 Molecular basis of bacterial outer membrane permeability revisited. *Microbiol. Mol. Biol. Rev.* **67**, 593-656.

- [5] Leive, L. 1965 Release of Lipopolysaccharide by EDTA treatment of *E. coli*. *Biochemical and biophysical research communications* **21**, 290-296.
- [6] Zhang, G., Meredith, T.C. & Kahne, D. 2013 On the essentiality of lipopolysaccharide to Gram-negative bacteria. *Current Opinion in Microbiology* **16**, 779-785. (doi:10.1016/j.mib.2013.09.007).
- [7] Bos, M.P. & Tommassen, J. 2005 Viability of a capsule-and lipopolysaccharide-deficient mutant of *Neisseria meningitidis*. *Infection and Immunity* **73**, 6194-6197. (doi:10.1128/IAI.73.9.6194).
- [8] Moffatt, J.H., Harper, M., Harrison, P., Hale, J.D.F., Vinogradov, E., Seemann, T., Henry, R., Crane, B., St. Michael, F., Cox, A.D., et al. 2010 Colistin resistance in *Acinetobacter baumannii* is mediated by complete loss of lipopolysaccharide production. *Antimicrobial Agents and Chemotherapy* **54**, 4971-4977. (doi:10.1128/AAC.00834-10).
- [9] Peng, D., Hong, W., Choudhury, B.P., Carlson, R.W. & Gu, X.X. 2005 *Moraxella catarrhalis* bacterium without endotoxin, a potential vaccine candidate. *Infection and Immunity* **73**, 7569-7577. (doi:10.1128/IAI.73.11.7569-7577.2005).
- [10] Steeghs, L., De Cock, H., Evers, E., Zomer, B., Tommassen, J. & Van der Ley, P. 2002 Outer membrane composition of a lipopolysaccharide-deficient *Neisseria meningitidis* mutant. *EMBO Journal* **20**, 6937-6945. (doi:10.1093/emboj/20.24.6937).
- [11] Steeghs, L., den Hartog, R., den Boer, a., Zomer, B., Roholl, P. & van der Ley, P. 1998 Meningitis bacterium is viable without endotoxin. *Nature* **392**, 449-450. (doi:10.1038/33046).
- [12] Osborn, M.J., Gander, J.E. & Parisi, E. 1972 Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. Site of synthesis of lipopolysaccharide. *J Biol Chem* **247**, 3973-3986.
- [13] Kamio, Y. & Nikaido, H. 1976 Outer membrane of *Salmonella typhimurium*: accessibility of phospholipid head groups to phospholipase c and cyanogen bromide activated dextran in the external medium. *Biochemistry* **15**, 2561-2570.
- [14] May, J.M., Simpson, B.W., Sherman, D.J., Ruiz, N. & Kahne, D. 2015 Lipopolysaccharide transport to the cell surface: periplasmic transport and assembly into the outer membrane. *Phil. Trans. R. Soc. B* **x**, xx-xx.
- [15] Glauert, a.M. & Thornley, M.J. 1969 The topography of the bacterial cell wall. *Annual review of microbiology* **23**, 159-198. (doi:10.1146/annurev.mi.23.100169.001111).
- [16] Kellenberger, E. & Ryter, a. 1958 Cell wall and cytoplasmic membrane of *Escherichia coli*. *The Journal of biophysical and biochemical cytology* **4**, 323-326.
- [17] Bladen, H.A. & Mergenhagen, S.E. 1964 Ultrastructure of *Veillonella* and Morphological Correlation of an Outer Membrane with Particles Associated with Endotoxic Activity. *J. Bacteriol.* **88**, 1482-1492.
- [18] Muhlradt, P.F. & Golecki, J.R. 1975 Asymmetrical distribution and artifactual reorientation of lipopolysaccharide in the outer membrane bilayer of *Salmonella typhimurium*. *Eur J Biochem* **51**, 343-352.
- [19] Osborn, M.J., Gander, J.E., Parisi, E. & Carson, J. 1972 Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. Isolation and characterization of cytoplasmic and outer membrane. *J Biol Chem* **247**, 3962-3972.
- [20] Miura, T. & Mizushima, S. 1968 Separation by density gradient centrifugation of two types of membranes from spheroplast membrane of *Escherichia coli* K12. *Biochimica et biophysica acta* **150**, 159-161.
- [21] Muhlrad, P.F., Menzel, J., Golecki, J.R. & Speth, V. 1973 Outer membrane of salmonella- Sites of export of newly synthesised lipopolysaccharide on the bacterial surface. *European journal of biochemistry / FEBS* **35**, 471-481.
- [22] Raetz, C.R. & Whitfield, C. 2002 Lipopolysaccharide endotoxins. *Annu Rev Biochem* **71**, 635-700. (doi:10.1146/annurev.biochem.71.110601.135414 110601.135414 [pii]).
- [23] Rocchetta, H.L., Burrows, L.L. & Lam, J.S. 1999 Genetics of O-antigen biosynthesis in *Pseudomonas aeruginosa*. *Microbiology and molecular biology reviews : MMBR* **63**, 523-553.
- [24] Karow, M. & Georgopoulos, C. 1993 The essential *Escherichia coli* *msbA* gene, a multicopy suppressor of null mutations in the *htrB* gene, is related to the universally conserved family of ATP-dependent translocators. *Molecular microbiology* **7**, 69-79.

- [25] Clementz, T., Bednarski, J.J. & Raetz, C.R.H. 1996 Function of the *htrB* high temperature requirement gene of *Escherichia coli* in the acylation of lipid A: HtrB catalyzed incorporation of laurate. *Journal of Biological Chemistry* **271**, 12095-12102. (doi:10.1074/jbc.271.20.12095).
- [26] Clementz, T., Zhou, Z. & Raetz, C.R.H. 1997 Function of the *Escherichia coli* *msbB* gene, a multicopy suppressor of *htrB* knockouts, in the acylation of lipid A. Acylation by MsbB follows laurate incorporation by HtrB. *Journal of Biological Chemistry* **272**, 10353-10360. (doi:10.1074/jbc.272.16.10353).
- [27] Polissi, A. & Georgopoulos, C. 1996 Mutational analysis and properties of the *msbA* gene of *Escherichia coli*, coding for an essential ABC family transporter. *Molecular microbiology* **20**, 1221-1233. (doi:10.1111/j.1365-2958.1996.tb02642.x).
- [28] Zhou, Z., White, K.a., Polissi, A., Georgopoulos, C. & Raetz, C.R.H. 1998 Function of *Escherichia coli* MsbA, an essential ABC family transporter, in lipid A and phospholipid biosynthesis. *Journal of Biological Chemistry* **273**, 12466-12475. (doi:10.1074/jbc.273.20.12466).
- [29] Doerrler, W.T., Gibbons, H.S., Christian, R. & Raetz, H. 2004 MsbA-dependent translocation of lipids across the inner membrane of *Escherichia coli*. *Journal of Biological Chemistry* **279**, 45102-45109. (doi:10.1074/jbc.M408106200).
- [30] Doerrler, W.T. & Raetz, C.R.H. 2002 ATPase activity of the MsbA lipid flippase of *Escherichia coli*. *Journal of Biological Chemistry* **277**, 36697-36705. (doi:10.1074/jbc.M205857200).
- [31] Ward, A., Reyes, C.L., Yu, J., Roth, C.B. & Chang, G. 2007 Flexibility in the ABC transporter MsbA: Alternating access with a twist. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 19005-19010. (doi:10.1073/pnas.0709388104).
- [32] Moeller, A., Lee, S.C., Tao, H., Speir, J.A., Chang, G., Urbatsch, I.L., Potter, C.S., Carragher, B. & Zhang, Q. 2015 Distinct Conformational Spectrum of Homologous Article Distinct Conformational Spectrum of Homologous Multidrug ABC Transporters. *Structure* **23**, 1-11.
- [33] Doshi, R. & van Veen, H.W. 2013 Substrate binding stabilizes a pre-translocation intermediate in the ATP-binding cassette transport protein MsbA. *The Journal of biological chemistry* **288**, 21638-21647. (doi:10.1074/jbc.M113.485714).
- [34] Reuter, G., Janvilisri, T., Venter, H., Shahi, S., Balakrishnan, L. & Van Veen, H.W. 2003 The ATP binding cassette multidrug transporter LmrA and lipid transporter MsbA have overlapping substrate specificities. *Journal of Biological Chemistry* **278**, 35193-35198. (doi:10.1074/jbc.M306226200).
- [35] Siarheyeva, A. & Sharom, F.J. 2009 The ABC transporter MsbA interacts with lipid A and amphipathic drugs at different sites. *The Biochemical journal* **419**, 317-328. (doi:10.1042/BJ20081364).
- [36] van Meer, G., Voelker, D.R. & Feigenson, G.W. 2008 Membrane lipids: where they are and how they behave. *Nature reviews. Molecular cell biology* **9**, 112-124. (doi:10.1038/nrm2330).
- [37] Bayer, M.E. 1968 Areas of Adhesion between Wall and Membrane of *Escherichia coli*. *Journal of General Microbiology* **53**, 395-404.
- [38] Kellenberger, E. 1990 The 'Bayer bridges' confronted with results from improved electron microscopy methods. *Molecular microbiology* **4**, 697-705.
- [39] Bayer, M.E. 1991 Zones of membrane adhesion in the cryofixed envelope of *Escherichia coli*. *Journal of structural biology* **107**, 268-280.
- [40] Chng, S.S., Gronenberg, L.S. & Kahne, D. 2010 Proteins required for lipopolysaccharide assembly in *Escherichia coli* form a transenvelope complex. *Biochemistry* **49**, 4565-4567. (doi:10.1021/bi100493e).
- [41] Freinkman, E., Okuda, S., Ruiz, N. & Kahne, D. 2012 Regulated Assembly of the Transenvelope Protein Complex Required for Lipopolysaccharide Export. *Biochemistry* **51**, 4800-4806. (doi:10.1021/bi300592c).
- [42] Okuda, S., Freinkman, E. & Kahne, D. 2012 Cytoplasmic ATP hydrolysis powers transport of lipopolysaccharide across the periplasm in *E. coli*. *Science* **338**, 1214-1217. (doi:10.1126/science.1228984).
- [43] Tefsen, B., Geurtsen, J., Beckers, F., Tommassen, J. & de Cock, H. 2005 Lipopolysaccharide transport to the bacterial outer membrane in spheroplasts. *J Biol Chem* **280**, 4504-4509. (doi:M409259200 [pii])

10.1074/jbc.M409259200).

- [44] Bos, M.P., Robert, V. & Tommassen, J. 2007 Biogenesis of the gram-negative bacterial outer membrane. *Annu Rev Microbiol* **61**, 191-214. (doi:10.1146/annurev.micro.61.080706.093245).
- [45] Malojčić, G., Andres, D., Grabowicz, M., George, A.H., Ruiz, N., Silhavy, T.J. & Kahne, D. 2014 LptE binds to and alters the physical state of LPS to catalyze its assembly at the cell surface. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 9467-9472. (doi:10.1073/pnas.1402746111).
- [46] Narita, S. & Tokuda, H. 2009 Biochemical characterization of an ABC transporter LptBFGC complex required for the outer membrane sorting of lipopolysaccharides. *FEBS Lett* **583**, 2160-2164. (doi:10.1016/j.febslet.2009.05.051).
- [47] Sherman, D.J., Lazarus, M.B., Murphy, L., Liu, C., Walker, S., Ruiz, N. & Kahne, D. 2014 Decoupling catalytic activity from biological function of the ATPase that powers lipopolysaccharide transport. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 4982-4987. (doi:10.1073/pnas.1323516111).
- [48] Ruiz, N., Gronenberg, L.S., Kahne, D. & Silhavy, T.J. 2008 Identification of two inner-membrane proteins required for the transport of lipopolysaccharide to the outer membrane of *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 5537-5542. (doi:10.1073/pnas.0801196105).
- [49] Sperandio, P., Cescutti, R., Villa, R., Di Benedetto, C., Candia, D., Deho, G. & Polissi, A. 2007 Characterization of *lptA* and *lptB*, two essential genes implicated in lipopolysaccharide transport to the outer membrane of *Escherichia coli*. *J Bacteriol* **189**, 244-253. (doi:JB.01126-06 [pii] 10.1128/JB.01126-06).
- [50] Tran, A.X., Dong, C. & Whitfield, C. 2010 Structure and functional analysis of LptC, a conserved membrane protein involved in the lipopolysaccharide export pathway in *Escherichia coli*. *J Biol Chem* **285**, 33529-33539. (doi:10.1074/jbc.M110.144709).
- [51] Chng, S.S., Ruiz, N., Chimalakonda, G., Silhavy, T.J. & Kahne, D. 2010 Characterization of the two-protein complex in *Escherichia coli* responsible for lipopolysaccharide assembly at the outer membrane. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 5363-5368. (doi:0912872107 [pii] 10.1073/pnas.0912872107).
- [52] Freinkman, E., Chng, S.S. & Kahne, D. 2011 The complex that inserts lipopolysaccharide into the bacterial outer membrane forms a two-protein plug-and-barrel. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 2486-2491. (doi:1015617108 [pii] 10.1073/pnas.1015617108).
- [53] Dong, H., Xiang, Q., Gu, Y., Wang, Z., Paterson, N.G., Stansfeld, P.J., He, C., Zhang, Y., Wang, W. & Dong, C. 2014 Structural basis for outer membrane lipopolysaccharide insertion. *Nature* **511**, 52-U569. (doi:10.1038/nature13464).
- [54] Qiao, S., Luo, Q., Zhao, Y., Zhang, X.C. & Huang, Y. 2014 Structural basis for lipopolysaccharide insertion in the bacterial outer membrane. *Nature* **511**, 108-U523. (doi:10.1038/nature13484).
- [55] Sperandio, P., Lau, F.K., Carpentieri, A., De Castro, C., Molinaro, A., Dehò, G., Silhavy, T.J. & Polissi, A. 2008 Functional analysis of the protein machinery required for transport of lipopolysaccharide to the outer membrane of *Escherichia coli*. *Journal of bacteriology* **190**, 4460-4469. (doi:10.1128/JB.00270-08).
- [56] Serina, S., Nozza, F., Nicastro, G., Faggioni, F., Mottl, H., Dehò, G. & Polissi, A. 2004 Scanning the *Escherichia coli* chromosome by random transposon mutagenesis and multiple phenotypic screening. *Research in microbiology* **155**, 692-701. (doi:10.1016/j.resmic.2004.05.006).
- [57] Sperandio, P., Pozzi, C., Dehò, G. & Polissi, A. 2006 Non-essential KDO biosynthesis and new essential cell envelope biogenesis genes in the *Escherichia coli* *yrbG-yhbG* locus. *Research in microbiology* **157**, 547-558. (doi:10.1016/j.resmic.2005.11.014).
- [58] Sperandio, P., Cescutti, R., Villa, R., Di Benedetto, C., Candia, D., Dehò, G. & Polissi, A. 2007 Characterization of *lptA* and *lptB*, two essential genes implicated in lipopolysaccharide transport to the outer membrane of *Escherichia coli*. *Journal of bacteriology* **189**, 244-253. (doi:10.1128/JB.01126-06).

- [59] Wu, T., McCandlish, A.C., Gronenberg, L.S., Chng, S.S., Silhavy, T.J. & Kahne, D. 2006 Identification of a protein complex that assembles lipopolysaccharide in the outer membrane of *Escherichia coli*. *Proc Natl Acad Sci U S A* **103**, 11754-11759. (doi:10.1073/pnas.0604744103).
- [60] Braun, M. & Silhavy, T.J. 2002 Imp/OstA is required for cell envelope biogenesis in *Escherichia coli*. *Molecular microbiology* **45**, 1289-1302.
- [61] Davidson, A.L., Dassa, E., Orelle, C. & Chen, J. 2008 Structure, function, and evolution of bacterial ATP-binding cassette systems. *Microbiology and molecular biology reviews : MMBR* **72**, 317-364, table of contents. (doi:10.1128/MMBR.00031-07).
- [62] Lima, S., Guo, M.S., Chaba, R., Gross, C.A. & Sauer, R.T. 2013 Dual molecular signals mediate the bacterial response to outer-membrane stress. *Science* **340**, 837-841. (doi:10.1126/science.1235358).
- [63] Chaba, R., Alba, B.M., Guo, M.S., Sohn, J., Ahuja, N., Sauer, R.T. & Gross, C.A. 2011 Signal integration by DegS and RseB governs the E-mediated envelope stress response in *Escherichia coli*. *Proc Natl Acad Sci U S A* **108**, 2106-2111. (doi:10.1073/pnas.1019277108).

Figure and table captions

Figure 1. Transport of LPS across the cell envelope. Following synthesis, LPS (lipid A-core oligosaccharide) molecules are flipped across the IM in an ATP-dependent manner by MsbA. At the outer leaflet, the O antigen is ligated to the outer core to form full-length LPS (not shown). LPS is transported across the periplasm and OM, and assembled at the cell surface by LptB₂FGCADE. LptB₂FG form an ABC transporter that utilizes ATP hydrolysis to extract LPS from the IM and push it along a periplasmic bridge built of homologous domains in LptCAD. It is unclear whether LptF, LptG, or both, interact with LptC. At the OM, the LptDE form a plug-and-barrel translocon that inserts LPS into the outer leaflet. Structural composition of *E. coli* LPS is shown on the left. Kdo=3-deoxy-D-manno-oct-2-ulosonic acid, Hep=L-glycero-D-manno-heptose, Etn=ethanolamine, P=phosphate, Glu=D-glucose, Gal=D-galactose.

Figure 2. MsbA undergoes structural conformational changes proposed to mediate LPS flipping. A. Crystal structures of MsbA (monomers in blue and cyan) suggest that the NBDs readily separate and dimerize in its inward facing state (PDB accession number: 3B5W, 3B5X) [31]. B. MsbA bound to a non-hydrolyzable ATP analog (AMPPNP in yellow spheres) shows an outward facing state and associated twisting of the transmembrane helices (PDB accession number: 3B5X, 3B5Y) [31].

Figure 3. Structures of LptB reveal site of interaction with TMD partners and conformational changes induced by ATP hydrolysis. A. Crystal structure of the LptB dimer (monomers in dark and light gray, nucleotides in yellow spheres) shows the groove (highlighted in brown) that is involved in interactions with LptFG at the membrane interface (symmetry mate derived from PDB accession number: 4P33) [47]. B. Superimposed structures of an ATP-bound (green) and an ADP-bound (blue) LptB monomer show conformational changes associated with ATP hydrolysis (PDB accession number: 4P32, 4P33)[47].

Additional Information

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Competing Interests

We have no competing interests.

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